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UNIVERSITÀ DEGLI STUDI DI TORINO

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1 **Translational efficiency of casein transcripts in Mediterranean river buffalo**

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ABSTRACT

15
16 Buffalo milk is characterized by the presence of all 4 casein fractions (α S1, β , α S2, and κ) encoded
17 by the 4 tightly linked autosomal genes (CSN1S1, CSN2, CSN1S2, and CSN3, respectively). In the
18 present paper, we report for the first time a quantitative characterization of buffalo casein transcripts
19 and show that the 4 genes are not transcribed and translated with the same efficiency. In particular,
20 the analysis of individual milk samples obtained from 9 Mediterranean river buffaloes showed that
21 the most abundant casein fractions were β (53.45%) and α S1 (20.61%), followed by α S2 and κ , at
22 14.28 and 11.66%, respectively. Quantification of the corresponding mRNA showed that the
23 percentage of transcripts of the 4 caseins was 16.48, 23.18, 55.87, and 4.47% for α S1, β , α S2, and κ ,
24 respectively. Translation efficiency was 0.25 for CSN1S2, 1.31 for CSN1S1, 2.39 for CSN2, and
25 2.69 for the CSN3 transcripts, respectively. A comparison of nucleotide sequences with the Kozak
26 consensus sequence was also carried out to investigate if the mRNA sequences might be responsible
27 for the observed differences.

28

29 **KEY WORDS:** Mediterranean river buffalo , casein , mRNA , quantification

30

SHORT COMMUNICATION

31

32 Buffalo milk is characterized by the presence of all 4 casein fractions (α S1, β , α S2, and κ) encoded
33 by 4 tightly linked autosomal genes (CSN1S1, CSN2, CSN1S2, and CSN3, respectively) mapped
34 on chromosome 7 (Iannuzzi et al., 2003). Today, the complete amino acid sequences of buffalo
35 casein (D'Ambrosio et al., 2008) are available, as well as the complete sequence and the relative
36 regulatory regions of genes encoding β - (Cosenza et al., 2009a) and κ -casein (Masina et al., 2007),
37 the 5'untranslated region (UTR), exon 1, and partial cDNA of the CSN1S1 gene (EMBL no.
38 GU593719, AF529305, AY948385, and AJ005430), and the sequences related to the cDNA as well
39 as short intronic sequences of the CSN1S2 gene (Cosenza et al., 2009b). Recently, Feligini et al.
40 (2009) developed a method for the quantification of α S1-, β -, α S2-, and κ -caseins in water buffalo

41 milk using reverse-phase HPLC. Unlike what has been accomplished for cattle, sheep, and goat
42 (Bevilacqua et al., 2006), no research has been carried out on the expression of casein genes in the
43 buffalo species as well as on their translational efficiency. The aim of this study was therefore to
44 assess the translation efficiency of the casein-encoding genes in Mediterranean river buffalo
45 through analysis of protein abundance and mRNA gene levels. For this purpose, individual raw
46 milk samples from 9 Mediterranean river buffaloes at comparable age, in third calving, at 120 d in
47 milking, belonging to the same farm located in province of Salerno (Italy), and free of clinical
48 mastitis were collected together with the monthly SCC controls by the local breeder association
49 (ANASB, Caserta, Italy). After collection, samples were immediately frozen and kept at -20°C
50 until analysis to prevent any proteolytic reaction induced by possible high SCC. Total RNA was
51 extracted from somatic cells (SCC range from 10,000 to 12,000/mL) present in the fresh milk by
52 using Nucleospin Blood and NucleoSpin Extract kits (Macherey-Nagel, Düren, Germany). The
53 quantity, quality, purity, and integrity of RNA after DNase treatment were estimated by means of
54 NanoDrop 2000c spectrophotometer (Thermo Scientific, Barrington, IL) and by electrophoresis on
55 a denaturing agarose gel. The reverse transcription was performed using Improm-II Reverse
56 Transcriptase (Promega, Madison, WI). By means of real-time quantitative PCR, the CSN1S1,
57 CSN2, CSN1S2, and CSN3 mRNA were quantified using standard curves, and the amount of each
58 transcript occurring within each sample was expressed as relative to the amount of transcript
59 measured for the single internal control (18S rRNA) used for normalization. Primer sequences for
60 CSN1S1, CSN2, CSN1S2, CSN3, and 18S rRNA amplification are reported in Table 1.

61 The results show that the transcript percentages were 16.48 (SD 4.99), 23.18 (SD 5.41), 55.87 (SD
62 8.22), and 4.47% (SD 0.96) for αS1 , β , αS2 , and κ , respectively. These values are significantly
63 different from those characterizing the transcripts of the same genes in cattle, sheep, and goats. In
64 fact, for these species, each casein transcript represents nearly 25% of the whole casein transcript
65 population (Bevilacqua et al., 2006). Although the starting material is different (milk somatic cells
66 in the present work and mammary gland cells in Bevilacqua et al., 2006), the results obtained by

67 different authors so far are comparable. In fact, different studies on goat and beef cows showed that
68 the relative amount of milk protein mRNA in milk somatic cells and mammary tissue samples is
69 highly correlated (Boutinaud et al., 2002; Murrieta et al., 2006).

70 The quantitative determination of the 4 casein fractions of the 9 individual buffalo milk samples
71 was carried out according to the method of Feligini et al. (2009). In the examined samples, β -
72 (19.81 g/L, SD 5.14) and α S1-casein (7.62 g/L, SD 2.52) were the major caseins, accounting for
73 53.45% (SD 6.63) and 20.61% (SD 4.29) of the whole casein fraction, respectively. On the contrary,
74 α S2- (4.99 g/L, SD 0.92) and κ -casein (4.25 g/L, SD 1.05) were less abundant, at 14.28% (SD 4.88)
75 and 11.66% (SD 2.26), respectively.

76 These results are in agreement with those found for buffalo by Feligini et al. (2009), but quite
77 different from those obtained for cattle, sheep, and goat, which show a percentage distribution of β -
78 and α S1-casein fractions of about 38% each (Bevilacqua et al., 2006). The observed differences in
79 the percentage distributions of the 4 casein fractions in buffalo milk and, particularly, of the β -
80 casein fraction, could account for its peculiar technological properties.

81 The ratio between the percentage of single milk protein fractions and the percentage of transcripts
82 produced in the mammary gland was estimated in order to evaluate the translation efficiency of the
83 buffalo gene casein transcripts. The values obtained show low translation efficiency (0.25, SD 0.07)
84 for the CSN1S2 transcripts, whereas the efficiency was higher for the CSN3, CSN2, and CSN1S1
85 transcripts: 2.69 (SD 0.74), 2.39 (SD 0.49), and 1.31 (SD 0.30), respectively. These results disagree
86 from those obtained by Bevilacqua et al. (2006) in cattle, goat, and sheep, in which the CSN2 and
87 CSN1S1 mRNA showed higher translational efficiency.

88 A comparison of nucleotide sequences with the Kozak consensus sequence was accomplished to
89 investigate if the mRNA sequences might be responsible for the observed differences. The context
90 of the translation initiation codon (AUG) plays an important role in determining the translation rate
91 (Kozak, 1991a,b). The Kozak consensus sequence occurs in eukaryotic mRNA and has the
92 sequence GCCGCCRCCAUGG, where R is a purine (adenine or guanine) 3 bases upstream of the

93 start codon (AUG), which is followed by another G (Kozak, 1987). The A nucleotide of the AUG
94 codon is referred to as number 1 and, although nucleotides -6, -3, and +4 are the most conserved
95 positions in natural mRNA sequences, it seems likely that other nearby nucleotides also contribute
96 to the translation process (Kozak, 1984; De Angioletti et al., 2004). In general, the more the
97 sequence around the initiation codon is homologous to the Kozak sequence (i.e., “strong”
98 consensus), the higher the efficiency of mRNA translation (Kozak, 1984).

99 The comparison of the sequence of the 4 transcripts in river buffalo with the Kozak sequence
100 showed that CSN1S1 had 4 positions out of 6 conserved in the GCCRCC consensus motif, whereas
101 CSN2, CSN3, and CSN1S2 had 3 homologous nucleotides (Table 2). In particular, 3 residues
102 directly upstream of the initiation codon were consecutive in CSN1S1 and CSN2 (-3, -2, and -1)
103 and 2 in CSN3 (-3 and -2). On the contrary, no conserved nucleotides were consecutive in
104 CSN1S2 (-6, -3, and -1; Table 2), which could account for the lower translational efficiency
105 recorded for the α S2-casein transcripts.

106 Concerning the higher translation efficiency of the κ -casein transcript compared with that observed
107 by Bevilacqua et al. (2006) for cattle, sheep, and goat, an explanation could be found through
108 comparison of the homologous messenger sequence among these species. The CSN3 mRNA in
109 buffalo, cattle, sheep, goat, mouse, rabbit, and pig have 2 consecutive AUG and, with the exception
110 of buffalo, in all these species, the first start codon shows a guanine in position -3 (Table 3).

111 It was proven (Kozak, 1984) that a start codon flanked by A in position -3 compared with G works
112 considerably better; that is, has higher translational activity. Therefore, the initiation sites may also
113 be designated as “strong” or “weak” based on this position. Ribosomes will initiate at the first AUG
114 codon to a limited extent even when the context is weak, but the poor context allows some
115 ribosomes to bypass the first AUG and reach a start codon further downstream, thus increasing the
116 complexity level of an organism by alternative gene expression pathways. This is called leaky
117 scanning (Kozak, 1984, 2005). Therefore, the presence of A in position -3 in the first start codon

118 could represent the optimal situation to ensure a more accurate and efficient translation of the
119 buffalo CSN3 transcript compared with that in other ruminants.

120 In conclusion, we report here for the first time a quantitative characterization of the buffalo casein
121 transcripts and show that the 4 mRNA are not transcribed and translated with the same efficiency.
122 Although the analysis of the sequences flanking the start codon can help to formulate hypotheses
123 concerning some of the observed differences in translation efficiency of the transcripts of the
124 investigated genes, other elements need to be analyzed to fully understand the mechanisms of
125 regulation of their expression. Based on results of different studies in higher eukaryotes and yeast, it
126 is generally accepted that not only the context of the translation initiation codon (AUG), but also the
127 length or alteration of the 5c UTR, secondary structure, GC content, upstream AUG codons or
128 upstream open reading frames, and the length of the 3c UTR play important roles in determining the
129 translation rate (Kozak 1991a,b; Tanguay and Gallie, 1996; Morris and Geballe, 2000; Koda et al.,
130 2004).

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134

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183

184 Table 1. Oligonucleotide primers sequence and positions

| Gene | Position, nucleotides | Primer sequence (5' to 3') ¹ | | EMBL accession no. | Amplicon size, bp |
|-----------------|------------------------------|---|----------------------|--------------------|-------------------|
| <i>CSN1S1</i> | 867–886 | Forward | TCTTCTTGAGTTCTCTACTG | AY948385 | 114 |
| | Complementary to 963–980 | Reverse | ACTCAGTGGCCTTTATAC | | |
| <i>CSN2</i> | 126–144 | Forward | AAGCCTTTCAAGCAGTGAG | FM946182 | 68 |
| | Complementary to 174–193 | Reverse | CCTCACTCTGAAACTTCTCA | | |
| <i>CSN1S2</i> | 574–591 | Forward | AAAATCAGCCAGCATTAC | FM865618 | 111 |
| | Complementary to 666–684 | Reverse | GGGAATAACGTTTGTCTTA | | |
| <i>CSN3</i> | 14100–14117 | Forward | TCAGTGAAACAGAGAAATAT | AM900443 | 106 |
| | Complementary to 14189–14205 | Reverse | GCTTTATTATGCAGGAA | | |
| <i>18S rRNA</i> | 1337–1352 | Forward | CGTTCTTAGTTGGTGG | NR_036642 | 76 |
| | Complementary to 1396–1412 | Reverse | GTAAGTACTAGTTAGCATGC | | |

185

186 ¹Primers were designed by means of Oligo 5.0 software (National Biosciences Inc., Plymouth, MN).

187

188 Table 2. Comparison of start codon flanking sequences of the 4 casein transcripts in the
 189 Mediterranean river buffalo

| Sequence ¹ | Position ² | | | | | | | | | |
|--------------------------|-----------------------|----------|----------|----------|----------|----------|-----------------|-----------------|-----------------|----------|
| | -6 | -5 | -4 | -3 | -2 | -1 | +1 | +2 | +3 | +4 |
| Kozak consensus sequence | G | C | C | R | C | C | <u>A</u> | <u>U</u> | <u>G</u> | G |
| <i>CSN2</i> | A | G | A | G | C | C | <u>A</u> | <u>U</u> | <u>G</u> | A |
| <i>CSN1S2</i> | G | U | A | A | A | C | <u>A</u> | <u>U</u> | <u>G</u> | A |
| <i>CSN1S1</i> | A | C | A | A | C | C | <u>A</u> | <u>U</u> | <u>G</u> | A |
| <i>CSN3</i> | G | G | U | A | C | A | <u>A</u> | <u>U</u> | <u>G</u> | A |

190

191 ¹Kozak consensus sequence = the optimal context for initiation of translation in mammals. *CSN2*,
 192 *CSN1S2*, *CSN1S1*, and *CSN3* are the genes encoding β -, α S2-, α S1-, and κ -casein, respectively.

193 ²The start codon (AUG) in the 4 casein transcripts is underlined; conserved nucleotides are shown
 194 in boldface.

195

196 Table 3. Comparison of start codon flanking sequences of the κ -casein transcripts in different
 197 species

| Species | EMBL accession no. | Position ¹ | | | | | | | | | | | | | |
|---------|-----------------------|-----------------------|----|----|----|----|----|----------|----------|----------|----|----|----|----|--|
| | | -6 | -5 | -4 | -3 | -2 | -1 | +1 | +2 | +3 | +4 | +5 | +6 | +7 | |
| Buffalo | AM900443 | G | G | U | A | C | A | <u>A</u> | <u>U</u> | <u>G</u> | A | U | G | A | |
| Cattle | AY380229 | G | G | U | G | C | A | <u>A</u> | <u>U</u> | <u>G</u> | A | U | G | A | |
| Sheep | NM_001009378 | G | G | U | G | C | A | <u>A</u> | <u>U</u> | <u>G</u> | A | U | G | A | |
| Goat | X60763 | G | G | U | G | C | A | <u>A</u> | <u>U</u> | <u>G</u> | A | U | G | A | |
| Pig | NM_001004026 | G | G | U | G | C | A | <u>A</u> | <u>U</u> | <u>G</u> | A | U | G | A | |
| Rabbit | Z18243 | G | G | U | G | C | A | <u>A</u> | <u>U</u> | <u>G</u> | A | U | G | A | |
| Rat | NM_007786 | G | G | U | G | C | A | <u>A</u> | <u>U</u> | <u>G</u> | A | U | G | A | |

198

199 ¹The first of 2 consecutive AUG codons is underlined.